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# IN THE UNITED STATES PATENT AND TRADE ARK OFFICE

Application of:

Carstens, Carsten-Peter

Serial No.:

09/492,590

Examiner:

G. Leffers Jr.

Filed:

January 27, 2000

Group:

1636

Entitled:

METHODS AND COMPOSITIONS FOR

HIGH LEVEL EXPRESSION OF A

HETEROLOGOUS PROTEIN WITH POOR.

CODON USAGE

Attorney Docket No.: 25436/1340 (Formerly 4114/85530)

Commissioner for Patents Washington, D.C. 20231

## DECLARATION UNDER 37 C.F.R. §1.131

#### I declare:

- 1. I, Carsten-Peter Carstens, am the inventor of the invention claimed in the above-noted U.S. patent application.
- 2. I have read and understood the Office Action mailed May 9, 2001, and have read and understood the cited reference, U.S. Patent No. 6,214,602 ("the '502 patent;" issued to Zdanovsky et al. on April 10, 2001, from an application filed August 28, 1998). I understand that the Examiner has cited the '602 patent as a novelty reference over claims 1-10, 15, 16, 19, 22-27, 32-40 and 42-44, and as an obviousness reference over claims 18, 20 and 21. The '602 patent is cited as teaching vectors and methods for the overproduction of Clostridium toxins and proteins by hosts such as E. coli. The methods taught by the '602 patent are said to feature the use of host cells containing a recombinant expression vector, wherein the expression vector encodes tRNAs that recognize rare codons and wherein the host cell expresses at least a fragment of at least one clostridial protein. The patent is also said to teach vector constructs that encode three tRNAs that recognize rarely used codons.
- 3. Prior to the August 28, 1998 filing date of the '602 patent, I had both conceived of and reduced to practice the claimed invention. The attached exhibits A and B, consisting of copies of my notebook entries detail the experiments that gave rise to the claimed invention, all performed before August 28, 1998. The dates on these exhibits have been reducted. For convenience, Appendices I and II contain transcripts of the relevant notebook entries.

A. Vector comprising an array of three or more tRNA genes which correspond to codons that are rarely used in a host cell

The first set of notebook entries, Exhibit A (transcripts in Appendix I), describes the development of pACYC-based vectors encoding genes for three recombinant tRNAs specific for rarely used codons. The first array is termed "RIL," for ArgU(R), ReY(I) and LeuW(L).

First, even the initial entry of Exhibit A (Entry # 1), describing experiments performed before August 28, 1998, shows that the vectors comprising an array of three tRNA genes were conceived of even before that date. That is, because Entry # 1 details efforts to ligate the RIL insert into the pACYC-LIC vector, I already had the critical RIL insert fragment and the intent to put it into an expression vector that would meet the limitations of the claims.

Second, Entry # 6 shows the ligation that generated pACTC-RIL vector constructs 49, 50 and 55, which were shown in the ensuing experiments (e.g., the PCR screen of Entry # 8 and the miniprep digests of Entry # 9) to contain the RIL insert, all performed before August 28, 1998. Thus, the vectors comprising an array of three or more tRNA genes which correspond to rarely used codons as claimed in the present application were reduced to practice as of the date of entry of Entry # 6, which is prior to August 28, 1998. The vectors resulting from the ligation were screened, isolated and confirmed by the date of Entry # 9, which is also before August 28, 1998. While it is clear that the vectors were made as of the date of Entry # 6 but not confirmed until the date of Entry # 9, both experiments were performed in advance of the August 28, 1998 priority date of the '602 patent.

B. Host cell containing a recombinant DNA molecule which comprises an array of three or more tRNA genes, wherein the tRNA genes correspond to codons that are rarely used in the host cell

The intent at the time of making the pACYC-RIL constructs was to introduce them to host cells for their influence on the expression of heterologous genes with rare codons. Thus, the conception of the claimed host cells that comprise an array of three or more tRNA genes, wherein the tRNA genes correspond to codon: that are rarely used in the host cell, necessarily preceded, or at a minimum, coincided with, the conception of the vectors themselves. Therefore, Exhibit A and Appendix I are also sufficient to demonstrate conception of the claimed host cells before August 28, 1998.

Similarly, Exhibit A and Appendix I are sufficient to demonstrate reduction to practice of host cells comprising an array of three or more tRNA genes, wherein the tRNA genes correspond to codons that are rarely used in the host cells. Specifically, the transformation of XL1-Blue host cells with the ligation reactions of Entry # 6 created host cells carrying such a construct. The PCR screening documented in Entry # 7 and the miniprep digests of Entry # 9 show that the pACYC-RIL clones 49, 50 and 55 are positive for the RIL insert. Finally, the test of Fip expression documented in Entries 10, 11 and 12 shows the introduction and induction of the pACYC-RIL construct in the well-

known protein expression E. coli host strain BL21(DE3). Each of these experiments was carried out before August 28, 1998.

In view of the above, host cells as claimed were conceived of and reduced to practice in advance of the August 28, 1998 priority date of the '602 patent.

# C. Method of producing a protein of interest

The second set of notebook entries, Exhibit B (transcript: in Appendix II), describes the use of the pACYC-RIL constructs to increase the production of a protein of interest from a gene with a non-E. coli codon bias.

The conception of a method of producing a protein from a gene with infrequently used codons, the method involving the expression of specific recombinant tRNAs from a vector in a host cell is necessarily very closely linked in time, if not coincident with or even prior to, the conception of the vector and host. Thus, conception of the method occurred before the performing the experiments described in the earliest entry in Exhibit A, which is prior to August 28, 1998. The reduction to practice is described below.

The hyperthermophilic archaeon *Pyrococcus furiosus* has a very AT-rich genome that results in distinctly different codon usage from the less AT-rich *E. coli* host cell genome. Therefore, one wishing to express *Pfu* DNA polymerase in *E. coli* will achieve only limited expression unless codon bias is compensated.

Exhibit B notebook Entry # 1 (page 48) shows the transformation of BL21(DE3)-RIL cells (corresponds to isolate 49) with a *Pfu* polymerase fusion protein. Entry # 2 shows a Coomassie-stained gel containing host cell proteins from *Pfu* polymerase construct-transformed cells with and without IPTG induction of IRNA construct expression. The gel photo shows the production of the *Pfu* polymerase fusion protein. The gel also shows, and the notebook entry states, that the production of the *Pfu* polymerase (rich in rare arg codons and Ile codons), is enhanced in RIL 49 host cells. Both experiments have been performed before August 28, 1998.

Exhibit B Entry # 2 therefore shows reduction to practice for a method of producing a protein of interest, comprising the step of culturing a host cell containing a recombinant DNA molecule that comprises an array of three or more tRNA genes, wherein the tRNA genes correspond to codons that are rarely used in the host cell, wherein the codons are present in the gene for the protein of interest, and wherein the conditions of culturing the host cell are sufficient to produce the protein of interest. The reduction to practice thus occurred prior to August 28, 1998.

4. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Date		

Carsten-Peter Carstens

# Appendix I Transcripts of notebook entries relating to vectors and host cells

(Note - several unrelated constructs were also being made in addition to the tRNA array constructs):

## Entry #1

[page 066]

## annealing of LIC-cloning reactions

I μI of LIC treated vector (pACYC-LIC or pESP<sub>4</sub>)
5 μI LIC-treated inserts (Flp, Ku70 and Ku80 and RIL)
4 μI dH2O

incubated for 1 h at RT

vectors setup: pESP4Ku70, pESP4Ku80, pESP4Flp and pACYC-Flp and RIL

used 1 µl to transform XL1-Blue cells. Plated transforms tions on LB-Amp (pESP4 clones) or LB-Cam plates (pACYC based).

### Entry #2

[page 067]

PCR Screening of pACYCFIp, pACYC-RIL, pESP4-Ku7), pESP4-Ku80 and pESP4-Flp colonies (Continuation of previous page) [details PCR reaction components and cycling conditions?]

[bottom of page 067, below gel photo]
None of the pACYC-RIL screened are positive.

#### Entry #3

[page 068]

Screening of more pACYC RIL clones (cont' from prev. page)
[details reaction setup and cycling conditions and shows diagnostic gel; no positive clones detected]

Confirmation diogests of RIL fragment used for LIC clon ng of pACYC-RIL and miniprep digest of selected ESP4Flp, ESP4 Ku80 and ESP4 Ku70 clones

[hottom of page, next to lower gel photo] test of RIL fragment

undigested:

610 bp

NcoI:

220 bp, 390 bp (high salt retards small frag. - NcoI uses 2X UB

(universal buffer))

XbaI:

180, 430 bp (+LIC sites)

the RIL fragment seems to be correct unclear why I can't clone - toxic?

## Entry #4

[page 080]

Ordered new primer for construction of RIL array. LIC cloning appeared to be very ineffective in previous attempts. Try to clone between the SpeI and XhoI sites of pACYC-LIC. Also ordered forward and reverse primer for RI and IL array to be cloned the same way.

### Entry #5

[page 084]

PCR-amplification of RIL. IL and RI fragments using primer with Spe/Xho extensions

[details reaction setup and cycling conditions and isolation of amplified fragments following gel electrophoresis]

### Entry #6

[page 086]

Construction of pACYC-RIL, pACYC-RI and pACYC-II.

- a) vector digest [details digest of pACYC-LIC with SpeI and XhoI]
- b) ligation [details ligation using 2  $\mu$ l of cut pACYC-LIC vector and 5 ml of RIL, RI or IL]

used 2 µl to transform XL1 Blue

#### Entry #7

[page 087]

PCR screen of pACYC-RIL and pACYC-IL (see prev. page)
[details reaction setup and cycling conditions and shows gel photo]

expected sizes: RIL 610 bp

IL 430 bp

pACYC-RIL: clone #7

pACYC-RI: 23, 25, 28, 29, 32, 33

#### Entry #8

[page 090]

minipreps of pACYC-RI and pACYC-RIL colonies (continuation of page 87)

expected fragment sizes 2192, 1230 (RI) or 1400 (RIL)

both pACYC-RI clones are confirmed by restriction digests.

None of the RIL clones is positive although they were PCR positive (comp. page 87)

[page 091]

Rescreened pACYC RIL colonies by PCR [details reaction setup and cycling conditions, shows gel photo]

none of the previous picked colonies are positive.

3 of the new colonies (49, 50 and 55) are positive.

## Entry #9

[page 091]

minipreps of pACYC-RIL clones (above)

expected

Ncol: 2192 bp, 1400 bp

Xbal:

All clones are positive by Ncol digests, but Xbal site seems to be missing (either from vector or from insert).

#### Entry #10

[page 095]

test of Flp expression in pACYC-RIL/BL21DE3 cells picked colonies from transformations on page 93 (one each expression clone in each RIL strain) and inoculated O/N cultures (1 ml Amp/Cam LB)

#### Entry #11

[page 095]

induction of Flp expression in BL21DE3/RIL strains [details growth and induction of expression of Flp]

Entry #12

[page 096]

detection of Flp expression in BL21DE3 RIL strains
[details gradient gel electrophoresis and Western blotting of samples from cells carrying RIL 49, RIL 50 and RIL 55 constructs]

Signal extremely strong. Had to add piece of paper between the membrane and the film to achieve reasonable exposure times (chemilum inogram next page). Took image with Eagle eye (signal strong enough for that).

# Appendix II Transcripts of notebook entries relating to production of a protein of interest

Entry #1

[page 48]

Comp IRL cells

[details making BL21DE3-RIL cells competent for transjormation]

transformed 100 µl ea. with

Pfu polymerase (CBP fusion)

} archaeal protein
} AT rich

Flavo PEF

CreProL#5

plated on LB Amp.

Entry #2

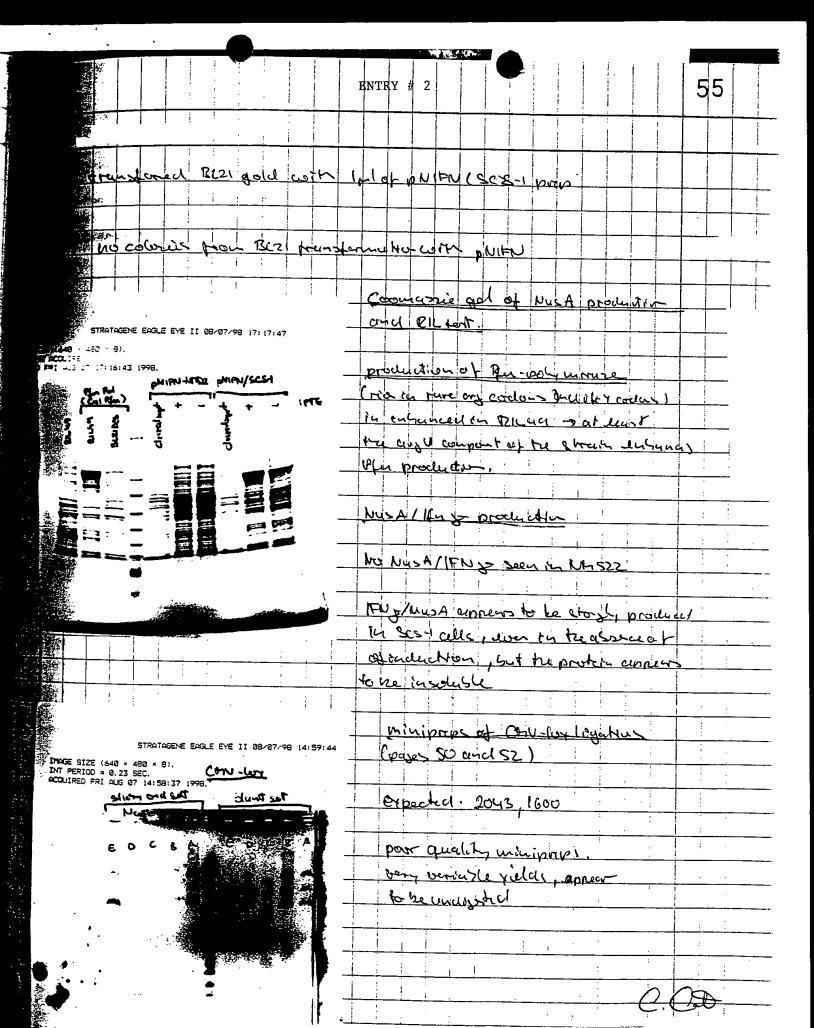
[page 55]

Coomassie gel of NusA production and RIL test.

production of Pfu-polymerase

(rich in rare arg codons and IleY codons) is enhanced in RIL49. At least the argU component of the strain enhances Pfu production.

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